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FINAL TECHNICAL REPORT

CONTRACT NO. DA-18-064-AMC-292(A)

WEST VIRGINIA UNIVERSITY

# THE LOCALIZATION OF INHALED PARTICULATE MATERIAL

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## Abstract

This investigation tried various ways of cutting and staining sections from lungs of animals exposed to a variety of aerosols containing particulates. The most successful approach involved the use of polystyrene latex spheres (Dow Chemical Company). These particles could be seen with phase microscopy on frozen sections of lung tissue. Anchoring of extracellular particles and definitive localization of particles remain problems as yet not solved by any of the modifications of standardized technics tried.

This investigation was an extension of Contract No. DA-18-064-AMC-76(A) and was concerned with the sequence of events occurring after inhalation of particulate material by experimental animals. Previous experience had exposed technical problems of a forbidding nature in an investigation using a morphological approach, the only type of attack permitted by our facilities. These problems included movement and loss of particles during routine histological preparation, poor visualization of tissue structure in unstained frozen sections and difficulty in identifying particles with certainty. Before abandoning the morphological approach to the problem, it was determined to pursue refinements of techniques that might result in accumulating valid data. The following experiments were directed toward finding a suitable identifiable particle and toward improving the efficacy of frozen sections.

## I. METHYL METHACRYLATE

Blocks of methyl methacrylate with Rhodamine B incorporated when irradiated with Cobalt 60 for 5 days are colored in incandescent light and also fluoresce. With the cooperation of the Chemical Engineering Department of West Virginia University, small particles of methyl methacrylate containing Rhodamine B were similarly treated. These particles fluoresced but the concentration of dye was too low to permit their visualization in incandescent light, thus not permitting their localization readily.

## II. BACILLUS GLOBIGII SPORES

Prior experiments attempting to stain Bacillus subtilis var. niger (B. globigii) spores with a fluorescein labelled antiserum prepared in our laboratory had been unsuccessful. A sample of conjugated antiserum against B. globigii prepared at Fort Detrick and shown to be effective with spores not in tissues and at pH 9 was made available to us. A series of guinea pigs exposed to aerosols containing heat killed B. globigii spores and sections of the lungs were exposed to this conjugated antiserum but none of the spores fluoresced. The experiment was repeated with the substitution of spores killed by treatment with 0.6% formalin but only a few spores fluoresced. The serum was sorbed with liver powder and the exposure repeated with no improvement in visualization of spores. Direct treatment of a suspension of spores on a glass slide with antiserum was followed by fluorescence of only approximately 1% of the spores.

tempts were made to stain formalin killed spores with Rhodamine B. Spores were exposed to a solution of dye for one half hour with very little fluorescence resulting. When the staining period was extended to overnight, the spores fluoresced on direct observation but not brightly enough to be located in tissue sections.

## III. FLUORESCENT PIGMENTS

In work performed before, fluorescent pigments which could be fractionated into suitable size ranges fluoresced quite brightly. Examination of tissues by incandescent light was unsatisfactory as the localization of the particles could not be ascertained. At that time, it was considered that better optical equipment than we were permitted to purchase under the terms of the contract might provide the resolution necessary to visualize the particles. Dr. John White of Fort Detrick kindly lent us a Zeiss binocular fluorescence microscope in January. Accordingly, repeat and reappraisal of the fluorescent pigment experiments were performed. Three guinea pigs were exposed for 10 minutes to an aerosol of Helecon pigment No. 2267 with a size range of 1-7.5 $\mu$ and an average diameter of 2.5 $\mu$ at a concentration of 1 X $10^8$ particles per cc. Animals were killed immediately post exposure, 24 hours post exposure and 5 days post exposure. Frozen sections of lung, lymph nodes and spleen were cut at 7 $\mu$ thickness and examined with the Zeiss fluorescence microscope. Sections of lymph nodes and spleen were unsatisfactory.

TABLE I

| Post exposure period | No. particles/lung section |
|---|---|
| 0 | 40 |
| 24 hours | 10 |
| 5 days | 2 |
| unexposed | 0 |

This trend although interesting is of no real significance because of the small number of animals. The visualization of the particles in incandescent light was not improved by the different optical equipment, so further experiments were not performed with this pigment.

## IV. LABELLING POLYSTYRENE LATEX BEADS

Attempts had previously been made to color polystyrene latex beads as received from Dow Chemical Company with various dyes. The results of these attempts were not satisfactory. Discussions were held with the Chemical Engineering Department of West Virginia University regarding a pilot attempt to render such particles both fluorescent and colored. It seemed easier to members of that department to incorporate suitable dyes during the fabrication of the particles and they attempted this in an

informal preliminary way. Samples of their results were sent to us for examination in May. These proved to be brightly fluorescent but were colorless in incandescent light and exhibited marked heterogeneity both in regard to size (1-30 $\mu$ in diameter) and shape. It would appear that more uniform fluorescent particles could be made comparable to those manufactured by Dow but would require more effort than could be expended by the Chemical Engineering Department on other than a contractual basis.

Polysciences, Inc., of Rydal, Pennsylvania, was consulted concerning the feasibility of double labelling polystyrene latex particles. Their response indicated that this was of project scope but could be attempted at an estimated cost of $1500. Since their reply was received about the middle of August, it was considered too late to pursue this somewhat tenuous lead.

## V. POLYSTYRENE LATEX BEADS AND PHASE MICROSCOPY

Since polystyrene latex beads as received from Dow Chemical Company, although uncolored, were excellent as to size and uniformity, efforts were made to use these as the test particle. It was found that these particles when viewed with phase microscopy could quite readily be identified. Determining the exact site of the particle has proven somewhat elusive. Unstained frozen sections do not yield sufficient detail even under phase to be certain of the exact position of the particles and whether they had moved from their original positions. Attempts were therefore made to refine the handling of tissue sections and to improve visualization.

### A. Staining of sections

Slides were coated with a 1% aquaeous solution of brilliant cresyl blue and were air-dried. Frozen sections of lungs from animals exposed to aerosols containing polystyrene latex beads were picked up by the slides in the hope that on thawing, the sections would become stained in the manner of a blood smear. The tissues were either too lightly stained to reveal detail or, when heavier layers of dried stain were used, visualization was impaired by particles of the dye.

A modified quick hematoxylin and eosin stain in which xylene was omitted yielded better results. This is subject to the criticisms that the liquids in the staining procedure may remove particles not firmly anchored and the clearing effect of the xylene is absent.

### B. Clearing agents

Since xylene dissolves polystyrene latex, other clearing agents were tested. Benzene, toluene, and chloroform are unsuitable for the same reason. Cedarwood oil does not dissolve the particles but it does not dry readily and is therefore impractical.

C. Mounting media

Permount, Ferrant's mounting medium and gum arabic were tested, with gum arabic being the most satisfactory since this solution is miscible with both alcohol and water, a requisite when slides are mounted from absolute alcohol without intervening xylene.

D. Anchoring of particles

1. Celloidin

Tissue sections were picked up on slides layered with a thin coating of 7% celloidin. They were then fixed in 10% formalin vapors for 5 minutes and stained with hematoxylin and eosin. This approach did not prevent particle movement after adding mounting medium and cover slip. When the tissue on the slide was subjected to a mist of 7% celloidin from an airbrush, particles did not move but tissue detail was poor.

2. "Spracyte"

This spray containing fixatives was applied to the frozen blocks of tissue, but the isopropyl alcohol contained in it rapidly defrosted the block and made cutting sections impossible.

3. Air dried lungs

Attempts were made to anchor inhaled particles by air drying immediately after exposure. Two guinea pigs were exposed for ten minutes to a high concentration of 3.49 $\mu$ polystyrene latex beads. The lungs from one animal were air dried overnight and those of the other were stored under refrigeration. Similar notches were cut in the right lower lobe of each lung and 10 cc of saline were perfused through each pair of lungs by means of tracheal cannulae. The saline from the air dried lungs contained many particles but none were recovered from the normal lungs.

## VI. POLYSTYRENE LATEX BEADS IN FROZEN, FIXED SECTIONS

Since the various approaches outlined above did not result in a satisfactory technique, it was determined to try fixing the lung sections after cutting with a view to getting some information as to fate of particles, even though the experimental conditions were not ideal. Guinea pigs were exposed for ten minutes to an aerosol of 3.49 $\mu$ polystyrene latex beads at a concentration equivalent to No. 4 $BaSO_4$ standard. Animals were sacrificed immediately post exposure, after 6 hours, 24 hours, 48 hours, and one month. At least five serial sections were cut from each animal's right lower lobe, medial lobe, and left lower lobe and from a hilar lymph node. Each section, after being placed on the slide, was

permitted to remain in air for approximately one minute to facilitate sticking to the slide and then was fixed in liquid 10% formalin for approximately one hour, was stained by a rapid hematoxylin and eosin technique which omitted use of a clearing agent and was mounted in gum arabic. The slides were then examined for particles by systematically studying the section with a 40X phase objective. The results are recorded in Table II. These results are subject to criticism both on deficiency of technique and paucity of animals. Those animals sacrificed in early stages probably had greater numbers of particles extracellularly in lumina of bronchioles and in more distal spaces, many of which were washed out by the fixing and staining procedures. The absence of particles in the animal sacrificed at one month is provocative but this observation must be verified in a larger series. If it proves to be valid, attention must be focused on the period after 48 hours and before one month. Of additional interest are the few particles detected in hilar lymph nodes. It may be that an inert particle, incapable of germination as opposed to an anthrax spore, may merely pass through the lymph nodes to other sites. It would be of interest to subject the animal to multiple exposures over an extended time to determine the ultimate site(s) of concentration of particles.

## TABLE II

Animals exposed for 10 minutes to aerosol of 3.49 μ polystyrene latex beads (concentration equiv. # $4BaSO_4$ Standard)

| Post exposure | Lung | Hilar lymph node | Spleen | Liver |
|---|---|---|---|---|
| 0 | 54 (15)* | 0 (5) | --- | --- |
| 0 | 23 (15) | 0 (5) | --- | --- |
| 0 | 45 (15) | 1 (5) | --- | --- |
| 6 hrs. | 58 (18) | 1 (20) | 5 (7) | 0 (7) |
| 24 hrs. | 5 (15) | 0 (5) | --- | --- |
| 24 hrs. | 22 (15) | 0 (5) | --- | --- |
| 48 hrs. | 27 (15) | 0 (5) | --- | --- |
| 48 hrs. | 21 (15) | 5 (5) | --- | --- |
| 1 month | 0 (21) | 0 (7) | --- | 0 (7) |

*Parentheses indicate number of serial sections examined.

# SUPPLEMENTARY

# INFORMATION

"In conducting the research reported herin, the investigator(s) adhered to 'Guide for Laboratory Animal Facilities and Care' established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, NAS-NRC"